

Role of Ser216 in the mechanism of action of membrane-bound lytic transglycosylase B: Further evidence for substrate-assisted catalysis

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Abstract Lytic transglycosylases cleave the β -(1 \rightarrow 4)-glycosidic bond in the bacterial cell wall heteropolymer peptidoglycan between the *N*-acetylmuramic acid (MurNAc) and *N*-acetylglucosamine (GlcNAc) residues with the concomitant formation of a 1,6-anhydromuramoyl residue. Based on sequence alignments, Ser216 in *Pseudomonas aeruginosa* membrane-bound lytic transglycosylase B (MltB) was targeted for replacement with alanine to delineate its role in the enzyme's mechanism of action. The specific activity of the Ser216 \rightarrow Ala MltB derivative was less than 12% of that for the wild-type enzyme, while its substrate binding affinity remained virtually unaltered. These data are in agreement with a role of Ser216 in orienting the *N*-acetyl group on MurNAc at the -1 subsite of MltB for its participation in a substrate-assisted mechanism of action.

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1. Introduction

The generally accepted model for the biosynthesis of the bacterial cell wall heteropolymer peptidoglycan invokes enzyme complexes comprised of both lytic transglycosylases (LTs) and transferases, the latter being a collection of penicillin-binding proteins (PBPs) [1]. The high-molecular weight PBPs catalyze the incorporation of the newly synthesized and translocated peptidoglycan precursor molecule, lipid II, into the existing sacculus at sites made available through the action of LTs [2,3]. While much effort has been made to understand the function and mechanism of action of the PBPs, the LTs have attracted considerably less attention.

The LTs are a class of bacterial autolysin that function to cleave peptidoglycan at the same site as lysozyme (E.C. 3.2.1.17; peptidoglycan *N*-acetylmuramoyl hydrolase; muramidase), specifically the β -(1 \rightarrow 4)-glycosidic bond between the *N*-acetylmuramic acid (MurNAc) and *N*-acetylglucosamine (GlcNAc) residues. However, LTs are catalytically distinct from the hydrolytic lysozymes (because they cleave peptidoglycan with the concomitant formation of 1,6-anhydro MurNAc residues [4]). These enzymes contribute to the metabolism of peptidoglycan, but their exact role has not been determined [5,6]. However, it has been demonstrated that the 1,6-anhydromuropeptide products are involved in the pathobiology of bacterial infections [7–9].

LTs appear to be ubiquitous in the eubacteria that produce peptidoglycan (viz. all but the cell wall-less mycoplasmas) [10]. Our laboratory has been engaged in the biochemical characterization of LTs from Gram-negative organisms, and in particular *Pseudomonas aeruginosa*. We have provided a kinetic characterization of the family 3 membrane-bound lytic transglycosylase B (MltB) [11] and the first report of substrate affinity for this class of enzyme [12]. Work with the β -hexosaminidase inhibitor NAG-thiazoline [13] has suggested that the LT mechanism employs substrate-assisted catalysis [14,15], while a genetic engineering study demonstrated the importance of both Arg187 and Arg188 with substrate recognition and binding at subsite -1 of the active site cleft [16]. This latter study also suggested the potential importance of the stem peptide of substrate in catalysis, possibly involving the highly conserved Ser216 residue.

Here, we further our characterization of an engineered, soluble derivative of *P. aeruginosa* MltB (sMltB) by investigating the role in catalysis of Ser216 in the -1 subsite of its active site cleft. Site directed mutagenesis was employed to replace Ser216 with Ala and the generated enzyme derivative was characterized kinetically.

2. Materials and methods

2.1. Chemical reagents and enzymes

Complete EDTA-free protease inhibitor tablets, glycine, and isopropyl-(β)-D-thiogalactoside (IPTG) were purchased from Roche Molecular Biochemicals (Laval, PQ). Ni-NTA resin was obtained from Qiagen (Valencia, CA) while the Source 15S column was supplied by Pharmacia Biotech (Baie d'Urfé, PQ). All other chemicals were purchased from either Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Nepean, ON), and were of reagent grade or HPLC-grade where appropriate.

Peptidoglycan was isolated and purified from *P. aeruginosa* PA01 as previously described [17]. The isolated peptidoglycan was treated with

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Abbreviations: GlcNAc, *N*-acetylglucosamine; HPAEC, high pH anion-exchange chromatography; LT, lytic transglycosylase; MltB, membrane-bound lytic transglycosylase B; MurNAc, *N*-acetylmuramic acid; PBP, penicillin-binding protein; PED, pulsed electrochemical detection; sMltB, soluble derivative of membrane-bound lytic transglycosylase B

DNase, RNase, and pronase and re-isolated by centrifugation as described by Glauner [18].

2.2. Bacterial strains and growth conditions

Bacterial strains used in this study were *Escherichia coli* DH5 (Gibco/BRL, Burlington, ON) for cloning and *E. coli* BL21(DE3) (Novagen, Madison, WI) for protein expression. Cultures were routinely grown in Luria–Bertani (LB; 1% tryptone peptone, 1% NaCl, and 0.5% yeast extract) broth or LB-agar plates at 37 °C. For protein expression experiments, cells were grown in Super Broth (3.2% tryptone peptone, 0.5% NaCl, and 2% yeast extract) at either 15 °C or 37 °C, as appropriate [11]. When necessary, growth media were supplemented with antibiotics at the following concentrations: kanamycin (Km; 30 $\mu\text{g mL}^{-1}$) or chloramphenicol (Cam; 34 $\mu\text{g mL}^{-1}$).

2.3. Site-directed mutagenesis

The Quick-Change (site-directed mutagenesis) system (Stratagene, LaJolla, CA) was used to engineer the *sMltB* gene encoding the single Ser216 \rightarrow Ala replacement within sMltB. Conditions for the mutagenesis PCR were those described by the manufacturer using PfuTurbo DNA polymerase. Plasmid pNBAC54-1 [11] was used as template in the PCR using the following primers: 5'-CGCTGGCCCGCGGCGTGCCTATGCCGCGC-3' and 5'-CGTAGCCCATGGCGCCGcgaTAGGCACCGC-3', with the bases in lower case and underlined representing those changed to introduce the desired mutation.

2.4. Isolation and purification of sMltB

The production and purification to apparent homogeneity of both the wild-type *P. aeruginosa* sMltB and its Ser216 \rightarrow Ala mutant derivative from appropriate *E. coli* transformants were conducted as previously described [11].

2.5. Assay for LT activity

The specific activity of purified sMltB and its Ser216 \rightarrow Ala derivative was measured using the assay developed by Blackburn and Clarke [19]. Briefly, sMltB was incubated at 37 °C in the presence of purified peptidoglycan suspended in 50 mM sodium acetate buffer, pH 5.8 containing 0.1% Triton X-100. At appropriate time intervals, samples were flash frozen at -78 °C to halt the reaction. The insoluble peptidoglycan was removed from the thawed samples by centrifugation (18,000 \times g, 20 min, 4 °C), and the recovered supernatants containing the released and soluble muropeptides were evaporated to dryness. The dried samples were hydrolyzed with 5.8 M HCl for 2 h at 98 °C and evaporated to dryness. The glucosamine content of the hydrolyzed samples was measured by high pH anion-exchange chromatography (HPAEC) using a PA1 pellicular anion-exchange column (4 \times 250 mm) with pulsed electrochemical detection (PED) (Dionex, Oakville, ON) [17]. For the analysis of muropeptide profiles of enzyme digests, insoluble peptidoglycan was removed from reaction mixtures by centrifugation (18,000 \times g, 20 min, 4 °C) and the supernatants were analyzed by HPAEC as previously described [11]. The data are presented as the average of at least three trials (\pm S.D.) using different preparations of enzymes.

2.6. Zymography

Zymograms involving the suspension of purified peptidoglycan in 12.5% polyacrylamide gels to a final concentration of 0.1% were performed as previously described [20–22].

2.7. Peptidoglycan binding assay

The binding of wild-type sMltB and the Ser216 \rightarrow Ala derivative to insoluble *P. aeruginosa* peptidoglycan was analyzed using a procedure described by Ursinus et al. [16,23] with modifications. Following incubation, insoluble peptidoglycan was recovered by centrifugation (18,000 \times g, 20 min, 4 °C), and the retained supernatants were analyzed by SDS–PAGE with Western immunoblot detection using an anti-His monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Relative intensities of the protein bands were determined with a GS-800 densitometer using the Quantity-One software package (Bio-Rad Laboratories).

2.8. Difference UV absorbance spectroscopy

Difference absorbance spectra of purified sMltB and its Ser216-Ala derivative were obtained as previously described [12]. Briefly, UV absorbance scans (220–320 nm) of enzyme samples (6 μM) in 10 mM ammonium acetate buffer, pH 6.5 containing 100 mM NaCl were collected using a Beckman DU-530 spectrophotometer after the sequential addition of ligand and incubation for 90 s at room temperature. Each scan was corrected for dilution by multiplying with a correction factor $[(\sum V_t + V_i)/V_i]$, where V_t is the titration volume and V_i is the initial volume. Scans were normalized by subtraction of the scan at zero ligand concentration and the trough to peak height at $A_{287\text{ nm}} - A_{281\text{ nm}}$ and $A_{253\text{ nm}} - A_{276\text{ nm}}$ were plotted versus the total ligand concentration, and dissociation constants (K_d) were derived using a non-linear regression analysis with a two-site binding model (Microcal Origin 5.0).

2.9. Other analytical methods

SDS–PAGE was performed as described by Laemmli [24] and gels were stained with Coomassie Brilliant Blue R-250 as described by Bolag et al. [25]. Protein concentrations were measured using the BCA protein assay kit supplied by Sigma with BSA serving as the standard.

3. Results and discussion

3.1. Production and activity of Ser216 \rightarrow Ala sMltB

Based on previous protein engineering and inhibition studies with NAG-thiazoline, we demonstrated that the mechanism of action of the family 3 LT MltB from *P. aeruginosa* invokes substrate-assisted catalysis which proceeds through an oxazolinium intermediate involving the *N*-acetyl group on MurNAc [15]. For this to occur, the *N*-acetyl group would have to be appropriately oriented at subsite -1 of the active site cleft. Examination of the three-dimensional structure of *E. coli* Slt35 and an alignment of the amino acid sequences of the family 3 LTs (data not shown) permitted the identification of Ser216 in *P. aeruginosa* MltB as a potential candidate to favorably position the *N*-acetyl moiety for intramolecular participation. This residue was thus targeted for site-specific replacement with alanine and the effects on enzyme activity and substrate binding were investigated.

The genes encoding wild-type sMltB and its Ser216 \rightarrow Ala derivative were expressed in respective *E. coli* transformants, as confirmed by SDS–PAGE and Western blot analysis using an anti-His tag monoclonal antibody (data not shown). Both enzymes were purified to apparent homogeneity by a combination of affinity chromatography on Ni^{2+} -NTA agarose and cation-exchange chromatography on Mono S (data not shown). Care was taken to use new Ni^{2+} -NTA agarose and a thoroughly washed Mono S column for these purifications to preclude the possible contamination of the two proteins with each other.

Zymogram analysis of Ser216 \rightarrow Ala sMltB using insoluble *P. aeruginosa* peptidoglycan as substrate indicated that its cleavage activity was significantly reduced compared to the wild-type enzyme at the same protein concentration (Fig. 1). Using the HPAEC-based assay developed previously for LTs [11], the soluble muropeptides released from insoluble PG by the mutant form of sMltB were identified and found to be the same as those generated by the wild-type enzyme (Fig. 1). The amount of released muropeptides was quantified over a 30 min period [19], and the specific activity of Ser216 \rightarrow Ala sMltB was found to be 89% lower than that of the wild-type ($1.45 \pm 0.56 \text{ nmol min}^{-1} \text{ mg}^{-1}$ compared to $13.0 \pm 4.1 \text{ nmol min}^{-1} \text{ mg}^{-1}$). Unfortunately, this low level of

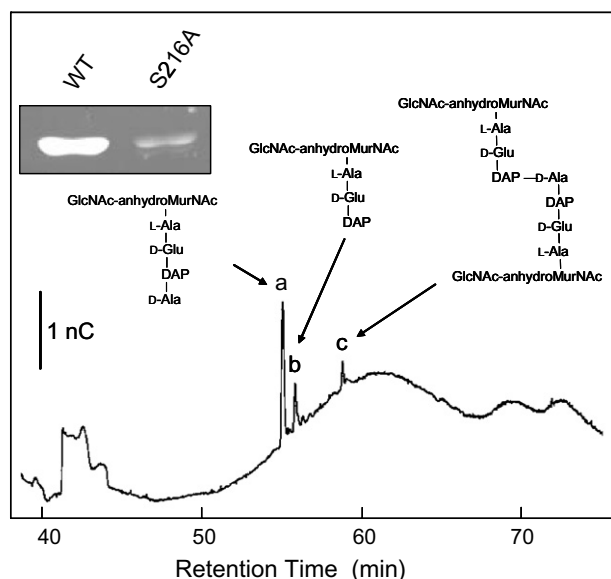


Fig. 1. Lytic activity of sMltB and its Ser216 → Ala derivative. Depicted is a representative HPLC profile of muropeptides generated by reaction of wild-type sMltB with *P. aeruginosa* PG at 37 °C for 15 min. HPLC conditions were as described in Section 2. The muropeptides identified were: (a) anhydro-disaccharide-tetrapeptide, (b) anhydro-disaccharide-tripeptide, and (c) cross-linked anhydro-disaccharide-tetrasaccharide. Inset: Zymogram of sMltB and mutant derivative using 0.1% (w/v) *P. aeruginosa* peptidoglycan as substrate. Both lanes were loaded with an equal amount of protein as determined by BCA protein assay.

Ser216 → Ala sMltB activity, combined with the inherent complexity of the assay and its associated large margins of error, precluded our ability to determine its Michaelis–Menten parameters with sufficient accuracy.

3.2. Substrate-binding capacity of Ser216 → Ala sMltB

To delineate the cause for the loss in catalytic activity, the wild-type and Ser216 → Ala sMltB were analyzed for their ability to bind insoluble peptidoglycan using an adsorption assay. Both enzymes (10 μg protein) were incubated on ice in the presence of 100 μg insoluble peptidoglycan. The insoluble material, including any bound protein, was removed by centrifugation and the pellet was washed free of any non-specifically bound proteins. Bound protein was extracted from the peptidoglycan by incubation in 2% SDS and all fractions were analyzed by SDS–PAGE. Using an anti-His tag monoclonal antibody in Western immunoblot assays, the percentage of bound protein was calculated based on the amount of protein

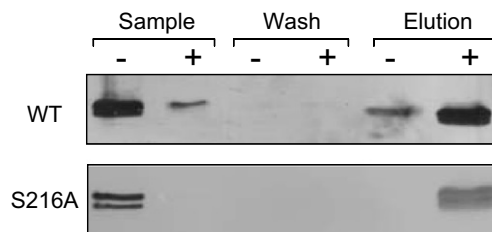


Fig. 2. Affinity assay of wild-type and Ser216 → Ala sMltB for insoluble peptidoglycan. Binding reactions were performed with approx. 10 μg protein and 100 μg *P. aeruginosa* PAO1 peptidoglycan (“+” lanes). To account for the non-specific adsorption of protein to the microfuge tubes, control experiments were performed that lacked added PG (“–” lanes). After incubation for 15 min on ice, PG was isolated (Sample), washed (Wash), and then extracted (Elution) with 2% SDS. Following each step, the PG was recovered by centrifugation and supernatants were subjected to SDS–PAGE and Western immunoblot analysis using the α-His probe.

remaining in the supernatant of the sample (containing peptidoglycan) and the control (no peptidoglycan). As can be seen in Fig. 2 and Table 1, the Ser216 → Ala mutant does not have an altered binding dissociation constant for insoluble peptidoglycan as compared to the wild-type enzyme.

Difference UV absorbance spectroscopy was also used to assess the ligand binding properties of Ser216 → Ala sMltB. As with the wild-type enzyme [12], the difference spectra of Ser216 → Ala sMltB showed a negative trough at 277 nm with a red shift upon titration with either GlcNAc–MurNAc–dipeptide or MurNAc–dipeptide (data not shown). The red shifts indicated a conformational change of an aromatic residue(s) to a more non-polar environment. Saturation curves of Ser216 → Ala sMltB titrated with these two ligands and transformation of the data provided K_d values which were very similar to those obtained with the wild-type enzyme (Table 1).

Taken together, these data suggest that replacement of Ser216 with Ala does not affect the binding of substrate and that the reduced specific activity observed with the mutant enzyme is thus likely caused by an effect on its catalytic mechanism. This would be expected if Ser216 was indeed involved in orienting the *N*-acetyl group on MurNAc at the –1 subsite for its subsequent attack on the anomeric center of the transition state to generate the putative oxazolinium intermediate.

4. Concluding remarks

With only one catalytic acid appropriately positioned at their active center, LTs are believed to catalyze the cleavage

Table 1
Affinity of sMltB and Ser216 → Ala derivative for peptidoglycan and components

Enzyme	Peptidoglycan-binding assay ^a		K_d (μM) by UV difference spectroscopy ^b	
	% Bound ^c	% Recovered ^d	MurNAc–diP	GlcNAc–MurNAc–diP
WT	93 ± 10	64 ± 16	52.3 ± 12	22.6 ± 0.31
S216A	90 ± 14	79 ± 10	70.3 ± 7.9	44.0 ± 0.84

^aThe binding assay was conducted as described in the legend to Fig. 2 (±S.D., $n = 3$).

^bDifference UV absorbance scans were obtained of the enzymes in 10 mM ammonium acetate buffer, pH 6.5 containing 100 mM NaCl titrated with either GlcNAc–MurNAc–dipeptide (GlcNAc–MurNAc–diP) or MurNAc–dipeptide (MurNAc–diP) at 25 °C.

^cCalculated by difference based on the amount of protein remaining in the supernatant after incubation with insoluble peptidoglycan relative to total protein applied.

^dBased on the amount of protein recovered in SDS extracts of peptidoglycan pellets relative to total protein originally applied.

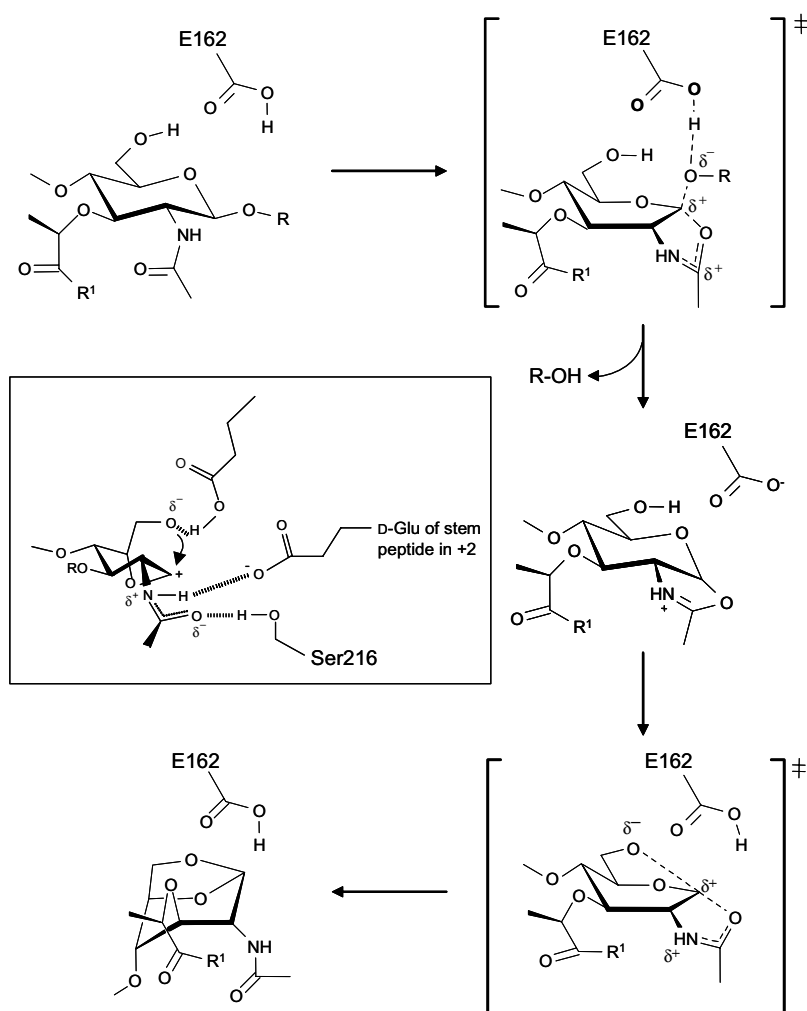


Fig. 3. Proposed catalytic mechanism of lytic transglycosylases. Inset: Ring distortion of oxocarbenium ion and role of Ser216 in orienting the *N*-acetyl group. R and R¹ denote a GlcNAc residue and stem peptide, respectively.

of peptidoglycan using substrate-assisted catalysis involving a mechanism which proceeds through an oxazolinium substrate intermediate (Fig. 3). In the LT Slt70 and a number of family GH-18 chitinases, two amino acid residues have been identified that coordinate the *N*-acetyl group of substrate in the –1 subsite and increase the nucleophilicity of its *N*-acetyl carbonyl [26–28]. With *E. coli* Slt70, Ser487 and Glu583, respectively, are thought to serve these functions. Evidence obtained in the current study suggests that Ser216 is involved in properly orienting the *N*-acetyl group on MurNAc for formation of the oxazolinium intermediate in *P. aeruginosa* sMltB. Family 3 LTs, however, appear to lack a homolog of the acidic residue at the –1 subsite. To accommodate for this apparent deficiency, van Asselt et al. [29] have proposed that the D-Glu residue of the stem peptide at the +2 subsite provides a functional substitution for the acidic residue on the enzyme. Indeed, they found that this free stem peptide in the +2 subsite could be modeled such that the free carboxyl of its D-Glu was in position to interact with the MurNAc *N*-acetyl group in the –1 subsite and thereby activate it for nucleophilic attack. This hypothesis is supported by our previous demonstration that the stem peptides on peptidoglycan substrates are important for binding to sMltB [16]. The observation that the MltBs from

both *E. coli* [30] and *P. aeruginosa* [11] have an absolute requirement for the stem peptide portion of peptidoglycan for activity further substantiates this postulate. However, a detailed investigation will be required to confirm the role of this neighboring stem peptide on the peptidoglycan in, what would be, a substrate-assisted–substrate-assisted mechanism of action.

Whereas we have argued that Ser216 participates in the mechanism of action of sMltB by helping to coordinate substrate at subsite –1, there are other possible explanations for the effect of its replacement on catalysis. For example, the replacement of Ser216 with Ala may have provoked a subtle conformational change in the active site to affect the local environment of Glu162 thereby lowering its effectiveness as the catalytic acid/base. Alternatively, Ser216 may indeed serve to properly orient the stem peptide of ligand at subsite –1 which in turn assists in the catalytic mechanism. At present, these possibilities cannot be excluded and structural studies would be required to confirm our hypothesis.

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